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In re Application of:

Sogabe et al.

Group Art Unit: 1652

Application No. 09/940,941

Examiner: Elizabeth Slobodyansky

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For: CREATINE AMIDINOHYDROLASE, PRODUCTION THEREOF AND USE THEREOF

DECLARATION UNDER 37 C.F.R. § 1.132 OF ATSUSHI SOGABE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Atsushi Sogabe, hereby declare that:

1. I am a citizen of Japan. I graduated in March of 1988 from the Laboratory of Applied Microbiology in the Department of Agricultural Chemistry at Shizuoka University in Shizuoka, Japan. I have been employed by Toyo Boseki Kabushiki Kaisha, the assignee with respect to the above-identified application, since April of 1988. As of July of 2003, I have been temporarily transferred to Toyobo Research Center Co., Ltd. I have been engaged in research in the fields of Enzymology and Molecular Biology. Accordingly, I am familiar with the knowledge of one of ordinary skill in the art of enzymes as of at least 1996.

2. I am one of the inventors named on U.S. Patent 6,080,553. I have read U.S. Patent 6,080,553, which I understand is related to the above-referenced reissue application.

3. U.S. Patent 6,080,553 pertains to creatine amidinohydrolases, which are described in various terms, including by reference to isoelectric point, in that patent.

4. The isoelectric point (pI) refers to the pH of a solution at which the total charges of an amphoteric electrolyte becomes 0 (i.e., neutral). The twenty different amino acids, which make up proteins, are amphoteric electrolytes having positive charges (e.g., amino group) or negative charges (e.g., carboxyl group). Each amino acid has a specific pI value. Accordingly, a protein composed of amino acids is an amphoteric electrolyte having a pI value unambiguously determinable experimentally or from the amino acid sequence of the protein.

5. U.S. Patent 6,080,553 provides the amino acid sequence of wild-type creatine amidinohydrolase derived from *Alcaligenes faecalis* in SEQ ID NO:1. Therefore, an ordinarily skilled artisan would be able to determine the pI value of the wild-type creatine amidinohydrolase by experiment and/or by calculation. The ability to do so was within the skill of an ordinarily skilled artisan since at least 1996.

6. U.S. Patent 6,080,553 describes the mutation of a wild-type creatine amidinohydrolase to create novel creatine amidinohydrolases with lower Km values. To preserve the inherent function and the physicochemical properties of the wild-type creatine amidohydrolase, the difference in the amino acid sequence between the source material and the mutants, or between different mutants, would have to be relatively minimal. Such small differences in the amino acid sequences of the mutants as compared to the wild-type amino acid sequence would not result in a significant difference in the pI value of the mutant creatine amidinohydrolases as compared to the wild-type enzyme.

7. One of ordinary skill in the art, reading U.S. Patent 6,080,553 in 1996, would have understood that U.S. Patent 6,080,553 describes three specific novel creatine amidinohydrolases, which can be obtained from the following deposited materials: *Escherchia coli* JM109 (pCRH273M2), *Escherchia coli* JM109 (pCRH273M1), and *Escherchia coli* JM109 (pCRH273M3). By comparison of Tables 2, 4, and 6 set forth in U.S. Patent 6,080,553, it is apparent that the majority of the physicochemical properties are conserved between the novel creatine amidinohydrolases, including the pI value.

8. One of ordinary skill in the art, reading U.S. Patent 6,080,553 in 1996, would have understood that U.S. Patent 6,080,553 also describes a group of novel creatine amidinohydrolases that include the three specific novel creatine amidinohydrolases disclosed in U.S. Patent 6,080,553.

9. If an ordinarily skilled artisan read U.S. Patent 6,080,553 in about 1996, the ordinarily skilled artisan would have recognized that these three specific novel creatine amidinohydrolases (described in U.S. Patent 6,080,553) are a representative subset of a group of novel creatine amidinohydrolases (also disclosed in U.S. Patent 6,080,553) with a shared set of physicochemical properties. The ordinarily skilled artisan also would have recognized that the pI value would be conserved among the members of this group of novel creatine amidinohydrolases as a function of conserving the function and physiological properties of the novel creatine amidinohydrolases.

10. If an ordinarily skilled artisan determined the actual pI value of the three specific novel creatine amidinohydrolases described in U.S. Patent 6,080,553 and found the actual pI value to be different from the pI value reported in U.S. Patent 6,080,553 for those three specific novel creatine amidinohydrolases, the ordinarily skilled artisan nevertheless would have understood that the pI value characterizing the group of novel creatine amidinohydrolases would be about the same as the actual determined pI value characterizing the three specific novel creatine amidinohydrolases that are members of that group. One of ordinary skill in the art would have had that understanding in 1996 and would have that same understanding today. Reading U.S. Patent 6,080,553 either in 1996 or today, one of ordinary skill in the art would believe that the inventors listed on U.S. Patent 6,080,553 had possession of the group of creatine amidinohydrolases with a pI value that was the same as the pI value of the three specific novel creatine amidinohydrolases described in U.S. Patent 6,080,553, even if the actual pI value for these three specific novel creatine amidinohydrolases differed from the pI value reported in U.S. Patent 6,080,553.

11. The pI values of the creatine amindinohydrolases described in Examples 4, 5, and 6 of U.S. Patent 6,080,553 were determined experimentally in the following manner. The three deposited bacterial strains that produce the creatine amidinohydrolases described in Examples 4, 5, and 6 of U.S. Patent 6,080,553 (i.e., *Escherichia coli* JM109 (pCRH273M1) deposited as FERM BP-5374, *Escherichia coli* JM109 (pCRH273M2)

deposited as FERM BP-5375, and *Escherichia coli* JM109 (pCRH273M3) deposited as FERM BP-5376, respectively) were obtained from the International Patent Organism Depository (IPOD), a Japanese International Depository Authority. Each bacterial strain was cultured in an L-broth (1.0% polypeptone, 0.5% yeast extract, 0.5% sodium chloride, pH 7.4) containing 100 μ g/ml ampicillin and 1.5% agar at 30° C for 24 hr. For a preculture, a part of the bacterial cells grown was transferred to 5 ml of an L-broth (pH 7.4) containing 100 μ g/ml ampicillin with an inoculating loop, and cultured with shaking at 30° C for 16 hr. Then, the obtained bacterial suspension (0.5 ml) was transferred to 50 ml of a creatine amidinohydrolase-producing medium I (1.2% polypeptone, 2.4% yeast extract, 0.4% glycerol, 1.25% di-potassium hydrogen phosphate, 0.23% potassium dihydrogen phosphate, and 200 μ g/ml ampicillin), and cultured with shaking at 30° C for 22 hr.

12. After the completion of the culture in the creatine amidinohydrolase-producing medium I, each bacterial suspension was centrifuged to recover bacterial cells, which were suspended in 20 mM potassium phosphate buffer (pH 7.5) and sonicated to extract creatine amidinohydrolase. The creatine amidinohydrolase was purified by polyethyleneimine treatment, ammonium sulfate fractionation, a heat treatment, DEAE-sepharose exchange column chromatography, and Octyl-Sepharose column chromatography. The purified creatine amidinohydrolase from each bacterial suspension gave a single band with SDS polyacrylamide gel electrophoresis.

13. *Alcaligenes faecalis* strain TE3581 described in Reference Example 1 of U.S. Patent 6,080,553 was cultured in an L-broth (1.0% polypeptone, 0.5% yeast extract, 0.5% sodium chloride, pH 7.4) containing 1.5% agar at 30° C for 47 hr. For preculture, a part of the bacterial cells grown was transferred to 5 ml of an L-broth (pH 7.4) with an inoculating loop and cultured with shaking at 30° C for 16 hr. Then, the obtained bacterial suspension (5 ml) was transferred to 500 ml of a creatine amidinohydrolase-producing medium II (0.65% creatinine, 1.2% polypeptone, 2.4% yeast extract, 0.4% glycerol, 1.25% di-potassium hydrogen phosphate, and 0.23% potassium dihydrogen phosphate), and cultured with shaking at 30° C for 48 hr.

14. After the completion of the culture in the creatine amidinohydrolase-producing medium II, the bacterial suspension was centrifuged to recover bacterial cells,

which were suspended in 20 mM potassium phosphate buffer (pH 7.5) and sonicated to extract creatine amidinohydrolase. The creatine amidinohydrolase was purified by polyethyleneimine treatment, ammonium sulfate fractionation, a heat treatment, DEAE-sepharose exchange column chromatography, and Octyl-Sepharose column chromatography. The purified creatine amidinohydrolase gave a single band with SDS polyacrylamide gel electrophoresis.

15. The pI value of each purified creatine amindinohydrolase described above, as well as CRH-211, a creatine amidohydrolase derived from *Actinobacillus sp.* (commercially available from Toyo Boseki K.K.), was determined using Fast System (Amersham Bioscience). pI Calibration Kit 3-10 (Amersham Bioscience) and pI Calibration Kit 2.5-6.5 (Amersham Bioscience) were used for the pI markers.

16. The pI values of the above-identified purified creatine amidinohydrolases were determined to be approximately 4.5, as reported in Figure 1 (see Attachment A).

17. Additionally, the pI values of creatine amidinohydrolases isolated from various other strains are known in the art to be approximately 4.5. For example, the pI value of the creatine amindinohydrolase from *Actinobacillus sp.* is 4.6 (see page 75 of "Creatine Amindinohydrolase from *Actinobacillus sp.*," Toyobo Co., Ltd. Catalog (Attachment B)). The pI value of the creatine amindinohydrolase of *Pseudomonas sp.* is 4.7 (see page 512, second column, last paragraph, of Yoshimoto et al., *Arch. Biochem. Biophys., 177,* 508-515 (1976) (Attachment C); and page 1 of "Creatine Amindinohydrolase from *Pseudomonas sp.*," Toyobo Co., Ltd. Catalog (Attachment D)). The pI value of the creatine amindinohydrolase of *Psuedomanas sp.* (expressed in recombinant *E. coli*) is 4.8 (see page 48 of "Creatinase, Pseudomonas species, recombinant," Roche Molecular Biochemicals 1999/2000 (Attachment E)). The pI value of the creatine amindinohydrolase of *Arthrobacter sp.* is 4.3 (see Abstract; page 3, first column, lines 2-12 and 17-30; and page 5, second column, lines 25-28, of JP 10-257890 (Attachment F)). The pI value of the creatine amindinohydrolase of *Paracoccus sp.* is 4.1 (see page 5, second column, line 37, of JP 7-274961 (Attachment G)). The pI value of the creatine amindinohydrolase of *Bacillus sp.* is 4.9 (see column 4, lines 10-12, of U.S. Patent 4,420,562 (Attachment H)). The pI value of the creatine amindinohydrolase of *Alcaligenes sp.* is 4.7 (see page 2157, penultimate paragraph, of Matsuda et al., *Chem. Pharm. Bull., 34(5),* 2155-2160 (1986) (Attachment I)).